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Molecular Genetics of the Maize (*Zea mays* L.) Aspartate Kinase-Homoserine Dehydrogenase Gene Family[1]

Gary J. Muehlbauer[2], David A. Somers, Benjamin F. Matthews, and Burle G. Gengenbach*

Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, Minnesota 55108 (G.J.M., D.A.S., B.G.G.); and United States Department of Agriculture, Agricultural Research Service, Plant Molecular Biology Laboratory, Beltsville, Maryland 20705–2350 (B.F.M.)

Aspartate kinase (AK) and homoserine dehydrogenase (HSDH) are enzymes in the aspartate-derived amino acid biosynthetic pathway. Recent biochemical evidence indicates that an AK-HSDH bifunctional enzyme exists in maize (*Zea mays* L.). In this report, we characterize three genes that encode subunits of AK-HSDH. Two cDNAs, pAKHSDH1 and pAKHSDH2, containing the full-coding sequence, and one partial cDNA, pAKHSDH3, encode amino acid sequences similar to the reported monofunctional AK and HSDH enzymes from prokaryotes and yeast (*Saccharomyces cerevisiae*) and to AK-HSDH bifunctional enzymes of prokaryotes, yeast, carrot (*Daucus carota*), and *Arabidopsis thaliana*. Immunological and biochemical analyses verify that the cDNAs encode AK-HSDH and indicate that both the AK and HSDH activities are feedback inhibited by threonine. RNA blots identify a 3.2-kb transcript in all maize tissues examined. pAKHSDH1 and pAKHSDH2 map to chromosomes 4L and 2S, respectively. This study shows that maize contains AK-HSDH bifunctional enzyme(s) encoded by a small gene family of at least three genes. Maize AK-HSDH has conserved sequences found in communication modules of prokaryotic two-component regulatory systems, which has led us to propose that maize AK-HSDH may be involved in a similar regulatory mechanism.

The Asp-derived amino acid pathway in plants and bacteria leads to the production of the essential amino acids: Lys, Thr, Met, and Ile (Bryan,1990a) (Fig. 1). The first enzyme in the pathway, AK (EC 2.7.2.4), catalyzes the phosphorylation of Asp to β-aspartyl phosphate. HSDH (EC 1.1.1.3), the third enzyme in this pathway, catalyzes the NADPH-dependent conversion of Asp 3-semialdehyde to homoserine. HSDH is the first committed reaction in the branch of the pathway that leads to Thr and Met. In plants, AK and HSDH are feedback inhibited by pathway end products (Bryan, 1990a). AK has been purified to near homogeneity and characterized in carrot (*Daucus carota*; Relton et al., 1988; Wilson et al., 1991) and maize (*Zea mays* L.; Dotson et al., 1989, 1990b; Azevedo et al., 1992a, 1992b). Three AK isoforms identified in higher plants are inhibited by Lys, Lys plus *S*-adenosyl Met, or Thr (Bryan, 1990a). In maize, all three AK isoforms were identified and characterized (Dotson et al., 1989; Azevedo et al., 1992a, 1992b). Lys-sensitive AK is a 255-kD heterotetramer consisting of subunits of 60 and 49 kD (Dotson et al., 1989, 1990b). Lys plus *S*-adenosyl Met-sensitive AK is relatively uncharacterized but appears to be structurally related to Lys-sensitive AK (Azevedo et al., 1992a). The existence of an AK-HSDH bifunctional enzyme in higher plants was first proposed for pea (*Pisum sativum*) (Aarnes and Rognes, 1974). For carrot and maize, AK activity was recently demonstrated to co-purify with HSDH activity (Wilson et al., 1991; Azevedo et al., 1992b), suggesting the presence of AK-HSDH in both dicotyledonous and monocotyledonous plants. Furthermore, a cDNA clone encoding carrot AK-HSDH (Weisemann and Matthews, 1993) and genomic and cDNA clones for *Arabidopsis* (Ghislain et al., 1994) have been isolated and characterized.

Maize AK-HSDH is a Thr-sensitive 180-kD bifunctional enzyme (Azevedo et al., 1992b). The bifunctional enzyme likely corresponds to the previously characterized Thr-sensitive HSDH in maize (Walter et al., 1979; Krishnaswamy and Bryan, 1986). Maize Thr-sensitive HSDH activity is conferred by a 180-kD homodimer composed of two 89-kD subunits; whereas Thr-insensitive HSDH activity is conferred by a 70-kD dimer of two 38-kD subunits and presumably represents an unrelated enzyme (Walter et al., 1979; Krishnaswamy and Bryan, 1986). It appears that the Thr-sensitive HSDH isolated previously probably contained AK activity. However, Thr-sensitive AK activity was not associated with the Thr-sensitive HSDH activity in maize until the report by Azevedo et al. (1992b). Thus, in maize it is likely that Thr-sensitive AK and HSDH compose the AK-HSDH bifunctional enzyme and that Lys-sensitive AK and Thr-insensitive HSDH are monofunctional AK and HSDH enzymes, respectively.

In this paper, we report the characterization of cDNA clones encoding subunits of an AK-HSDH bifunctional enzyme in maize. This is the first report characterizing these genes from a monocotyledonous plant. Molecular and biochemical evidence demonstrates that at least three cDNAs, pAKHSDH1, pAKHSDH2, and pAKHSDH3, encode AK-HSDH. pAKHSDH1 and pAKHSDH2 were mapped to chro-

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[2] Present address: Department of Plant Biology, 111 Koshland Hall, University of California-Berkeley, Berkeley, CA 94720.

* Corresponding author; fax 1–612–625–1268.

Abbreviations: AK, aspartate kinase; cM, centimorgan; HSDH, homoserine dehydrogenase; RFLP, restriction fragment length polymorphism.

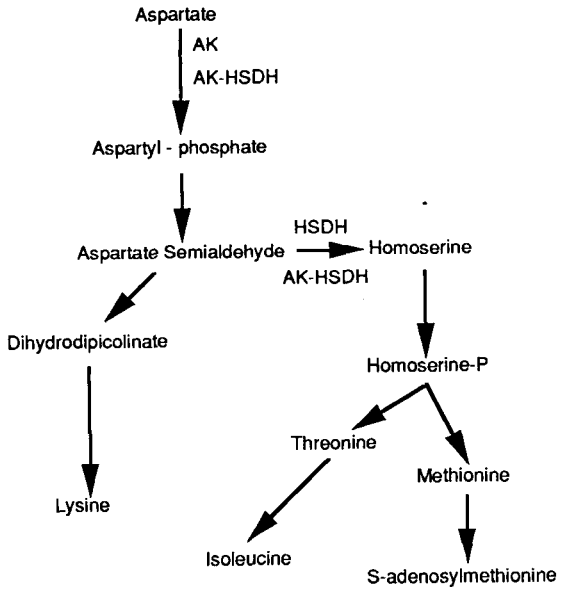

Figure 1. Abbreviated diagram of the Asp-derived amino acid pathway leading to the production of Lys, Thr, Met, and Ile. AK catalyzes the first reaction, HSDH catalyzes the third reaction, and the bifunctional AK-HSDH enzyme catalyzes both the first and the third steps in this pathway.

mosome 4S and 2L, respectively. In addition, a novel regulatory mechanism for AK-HSDH is proposed.

MATERIALS AND METHODS

Screening a Maize Seedling Leaf cDNA Library

A *Zea mays* L. λgt11 seedling leaf cDNA library of inbred line A188 was provided by S. Gantt (University of Minnesota). Plaques were transferred to Millipore[3] (Bedford, MA) membranes, prehybridized, and hybridized according to standard conditions (Sambrook et al., 1989), and 800,000 plaques were screened. The 1.8-kb *Eco*RI 5′ fragment (AK portion of the clone) from the carrot (*Daucus carota*) AK-HSDH cDNA (Weisemann and Matthews, 1993) was used as a probe. Positive plaques were isolated and purified, and DNA was isolated (Chisholm, 1989). Southern blot analysis of DNA digested with *Eco*RI indicated three different clones based on the size of the hybridizing bands. The cDNA inserts in the λ clones were digested with *Eco*RI and subcloned into the *Eco*RI site of pBluescriptII KS+ (Stratagene, LaJolla, CA) according to standard techniques (Sambrook et al., 1989).

Subcloning and Sequencing Procedures

pAKHSDH1 exists as two *Eco*RI subclones of 366 and 2812 bp. The larger *Eco*RI subclone contains three *Pst*I sites, which allowed the creation of four *Pst*I subclones into pBluescriptII KS+. These subclones were sequenced following the procedure provided with the Sequenase II sequencing kit (United States Biochemical Corp., Cleveland, OH) using T7 and T3 primers (Promega, Madison, WI). After preliminary sequencing, primers specific to pAKHSDH1 were synthesized and used to sequence the remainder of pAKHSDH1. pAKHSDH2 exists as two *Eco*RI subclones of 563 and 2482 bp. pAKHSDH3 exists as two *Eco*RI subclones of approximately 2000 and 600 bp. T3 and T7 primers were used initially to sequence the ends of the subclones from pAKHSDH2 and pAKHSDH3. The remainder of pAKHSDH2 was sequenced at the microchemical facility of the University of Minnesota.

Plant Material

Maize inbred line B73 was grown in the field at St. Paul in 1992 and hand pollinated. Embryos and endosperm tissue were collected from kernels 20 d after pollination for RNA isolations. The immortalized F_2 population (Gardiner et al., 1993) from the Co159 × Tx303 cross was grown in the field at St. Paul in 1992. For chromosome mapping, five immature ears from each of 55 F_2 lines were combined for DNA isolations to represent each F_2 line.

DNA Isolation and DNA Gel Blot Analysis

DNA was isolated from freeze-dried immature ears and 7-d-old leaf tissue (Saghai-Maroof et al., 1984). DNA was digested with restriction endonucleases following suppliers' instructions and electrophoresed overnight in 1× TAE (0.04 M Tris-acetate, 0.001 M EDTA), 0.8% (w/v) agarose gels. The DNA was transferred overnight onto Immobilon-N (Millipore), and blots were prehybridized and hybridized overnight at 65°C using standard conditions (Sambrook et al., 1989). Final posthybridization washes were at 65°C with 0.1× SSC and 0.1% (w/v) SDS.

RNA Isolation and RNA Gel Blot Analysis

Isolated B73 endosperms and embryos 20 d after pollination and Black Mexican Sweet suspension cells were frozen in liquid nitrogen, and poly(A)$^+$ mRNA was extracted using the FastTrack poly(A)$^+$ mRNA isolation kit (Invitrogen, San Diego, CA). Poly(A)$^+$ mRNA (2 μg) was denatured in glyoxal and DMSO (Sambrook et al., 1989) and electrophoresed in 10 mM sodium phosphate, pH 7.0, 1% (w/v) agarose gels. RNA was transferred overnight to Nytran membrane (Schleicher & Schuell), prehybridized, and hybridized overnight at 42°C in 50% (v/v) formamide (Sambrook et al., 1989). Final posthybridization washes were at 42°C with 0.5× SSC and 0.5% (w/v) SDS. For low stringency conditions, the RNA blots were prehybridized and hybridized overnight at 35°C in 50% (v/v) formamide, and washes were at room temperature with 2× SSC and 0.5% (w/v) SDS.

DNA Probes and Labeling Reactions

DNA probes from the carrot and maize cDNA clones were restriction enzyme digested or generated using the PCR (Sambrook et al., 1989) and isolated from agarose gels following

[3] Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture or the University of Minnesota and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

the procedure accompanying the Geneclean kit (Bio 101, LaJolla, CA). The probes were random-primer labeled with [α-^{32}P]dCTP (Feinberg and Vogelstein, 1983).

AK-HSDH Enzyme Isolation

Seven-day-old Black Mexican Sweet suspension cells (75 g) were ground in liquid nitrogen and then suspended in 750 mL of 100 mM Tris-HCl, pH 7.4, 2 mM Lys, 2 mM Thr, 200 mM KCl, 1 mM DTT, 2 mM EDTA, 0.1 mM PMSF, and 10% (v/v) ethylene glycol (buffer 1). The extract was centrifuged for 40 min at 10,000*g*. The supernatant was brought to 70% saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$-precipitated protein was centrifuged for 40 min at 10,000*g*, and the pellet was resuspended in 10 mM potassium phosphate, pH 7.5, 1 mM DTT, 2 mM Lys, 2 mM Thr, and 10% (v/v) ethylene glycol (buffer 2). The protein was desalted on a G-25 column in buffer 2 and loaded on a Blue Sepharose column with a flow rate of 0.3 mL/min. The column was washed in buffer 2 overnight, and the protein was eluted with 0.5 mM NADPH at the same flow rate. The Blue Sepharose fractions were stored in 10 mM potassium phosphate, pH 7.5, 1 mM DTT, 2 mM Lys, 2 mM Thr, and 10% (v/v) glycerol at −70°C for several months without loss of activity.

AK and HSDH Enzyme Assays

AK activity was assayed following the hydroxamate method (Hitchcock and Hodgson, 1976). HSDH was assayed following the oxidation of NADPH at 340 nm (Gengenbach et al., 1978). Inhibition properties of AK and HSDH were determined by conducting assays in 5 mM Thr and 5 mM Lys. Protein concentration was determined following the procedure of Bradford (1976).

Protein Gel Electrophoresis and AK and HSDH Activity Stains

Electrophoresis was conducted on 8 to 25% gradient nondenaturing and denaturing minigels using the Phastgel system (Pharmacia). HSDH activity was visualized in nondenaturing gels following the procedure of Walter et al. (1979). AK activity was visualized in nondenaturing gels according to the procedure of Dotson et al. (1989).

Antibody Production

For antibody production, a 13-amino acid peptide (DPDYLESEKRLEK) deduced from pAKHSDH1 was identified following the requirements for antigenicity (Van Regermortel et al., 1988). A Cys was added to the amino terminus of the sequence, and a 14-amino acid peptide (CDPDYLESEKRLEK) was synthesized (University of Minnesota, microchemical facility). The peptide (2 mg) was conjugated to keyhole limpet hemocyanin and BSA following the kit procedure (Pierce, Rockford, IL). Two rabbits were initially immunized with 250 μg of keyhole limpet hemocyanin-conjugated peptide emulsified in Freund's complete adjuvant. Rabbits were subsequently immunized with 125 μg of keyhole limpet hemocyanin-conjugated peptide emulsified in Freund's incomplete adjuvant every 10 to 14 d. Serum was collected before the initial immunization (preimmune serum) and 8 weeks after the first immunization (immune serum).

Protein Blotting

Protein was blotted onto Immobilon-P (Millipore) for 2 to 4 h via capillary action in 48 mM Tris, 39 mM Gly, and 20% (v/v) methanol (Towbin et al., 1979). Membranes were blocked in TBST (10 mM Tris, pH 7.4, 0.9% [w/v] NaCl, 0.5% [v/v] Tween 20) and 1% (w/v) BSA for 4 h. Antibody (preimmune and immune serum) was incubated with the western blots overnight at a 1000:1 dilution. The membranes were washed for 5 to 6 h with several changes of TBST. Goat anti-rabbit alkaline phosphatase (2000:1 dilution; Bio-Rad) was incubated with the membranes for 3 to 4 h. The membranes were washed for 1 h with several changes of TBST and then equilibrated in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM MgCl) for 10 min. Alkaline phosphatase activity was visualized according to the method of Harlow and Lane (1988).

Linkage Calculations and Mapping

The map locations of pAKHSDH1 and pAKHSDH2 were determined using the MAPMAKER (Lander et al., 1987) computer program with the University of Missouri core RFLP data base (Gardiner et al., 1993).

RESULTS

Isolation of AK-HSDH cDNAs and Sequence Analysis

Three different AK-HSDH cDNA clones were isolated from a maize seedling leaf cDNA library constructed in λgt11 using a carrot AK-HSDH cDNA as a probe. The three clones were determined to be different based on restriction enzyme mapping and sequence data. Two cDNAs containing the full-coding region, pAKHSDH1 and pAKHSDH2, were completely sequenced, whereas initial sequence data from pAKHSDH3 demonstrated that it did not contain the entire coding region of an AK-HSDH enzyme. Comparisons of the deduced amino acid sequences of pAKHSDH1 and pAKHSDH2 (deduced amino acid sequences referred to as AKI-HSDHI and AKII-HSDHII, respectively) and several AK, AK-HSDH, and HSDH enzymes from either carrot, *Arabidopsis*, yeast, or bacteria demonstrated significant sequence conservation (Table I). The amino acid identities suggested that the maize cDNAs encode AK-HSDH bifunctional enzymes. AKI-HSDHI and AKII-HSDHII are 88% identical at the amino acid level in the mature coding region. The regions of the partial cDNA pAKHSDH3 that were sequenced showed significant nucleotide identity with the corresponding regions of pAKHSDH1 and pAKHSDH2 (Table I), indicating that it is a member of the *ak-hsdh* gene family.

The sequence data indicate that the 3178-bp pAKHSDH1 contains a 41-bp 5′ untranslated sequence and a 377-bp 3′ untranslated region and encodes a 92-amino acid putative plastid transit peptide sequence and an 828-amino acid mature coding sequence (Fig. 2). pAKHSDH2 is 3045 bp in length and contains a 43-bp 5′ untranslated region and a 251-bp 3′ untranslated region and encodes an 89-amino acid

Table I. *Sequence comparisons of maize AK-HSDH with AK, HSDH, and AK-HSDH from other organisms*

Enzyme	Percentage Identity[a]		Refs.
	Z. mays AKI-HSDHI	*Z. mays* AKII-HSDHII	
Z. mays AKI-HSDHI			
Complete coding region		77	This paper
Mature protein		88	
Transit peptide		38	
Transit peptide, nucleotide level		60	
3′ untranslated, nucleotide level		50	
Z. mays pAKHSDH3			
Nucleotides 578–844	79	86	This paper
Nucleotides 2238–2481	80	92	
Nucleotides 2481–2725	81	93	
Nucleotides 2736–3008	50	66	
D. carota AK-HSDH			
Mature protein	77	75	Weisemann and Matthews, 1993
A. thaliana AK-HSDH			
Mature protein	75	75	Ghislain et al., 1994
E. coli AKI-HSDHI			
Mature protein	38	39	Katinka et al., 1980
AK domain	32	32	
Interface domain	39	40	
HSDH domain	43	44	
E. coli AKII-HSDHII			
Mature protein	31	32	Zakin et al., 1983
AK domain	30	27	
Interface domain	22	24	
HSDH domain	40	41	
E. coli AKIII			
AK domain	29	29	Cassan et al., 1986
S. marcescens AK-HSDH			
Mature protein	38	39	Omori et al., 1993
AK domain	32	31	
Interface domain	40	41	
HSDH domain	44	44	
Saccharomyces cerevisiae AK			
AK domain	27	27	Rafalski and Falco, 1988
S. cerevisiae HSDH			
HSDH domain	43	42	Thomas et al., 1993
Bacillus subtilus AKII			
AK domain	28	28	Chen et al., 1987

[a] Demonstrates amino acid identity unless otherwise indicated.

transit peptide sequence and an 828-amino acid mature coding sequence (Fig. 2). The calculated molecular masses for the subunits deduced from pAKHSDH1 and pAKHSDH2 were 90,319 and 89,818 D, respectively, allowing the possibility for homodimer or heterodimer formation of the holoenzyme. These values are very close to the estimated value of 89,000 D of Thr-sensitive HSDH as determined by SDS-PAGE (Walter et al., 1979; Krishnaswamy and Bryan, 1986).

Both AKI-HSDHI and AKII-HSDHII (deduced amino acid sequences from pAKHSDH1 and pAKHSDH2, respectively) have putative transit peptide sequences (Fig. 3), which are consistent with the proposed plastid localization of Thr-sensitive HSDH (Bryan et al., 1977). The presumed transit peptide lengths are based on comparisons to the start of the *Escherichia coli* and *Serratia marcescens* AKI-HSDHI enzymes (Fig. 3). The deduced amino acid sequences of the transit peptides exhibited 38% identity (Fig. 3, Table I). The lengths (92 and 89 amino acids for maize AKI-HSDHI and AKII-HSDHII, respectively) are within the range of other plastid transit peptide sequences (Keegstra et al., 1989; von Heijne et al., 1989) (Fig. 3). The amino acid composition of the proposed transit peptides is consistent with plastid transit peptides except for a higher number of charged amino acids (8 negatively charged and 17 positively charged for AKI-HSDHI; 7 negatively charged and 13 positively charged for AKII-HSDHII). However, the net positive charge of 9 and 6 for AKI-HSDHI and AKII-HSDHII, respectively (Fig. 3), is similar to typical plastid transit peptides (von Heijne et al., 1989). AKI-HSDHI and AKII-HSDHII also contain a lower percentage of hydroxylated amino acids (16.3 and 12.3%,

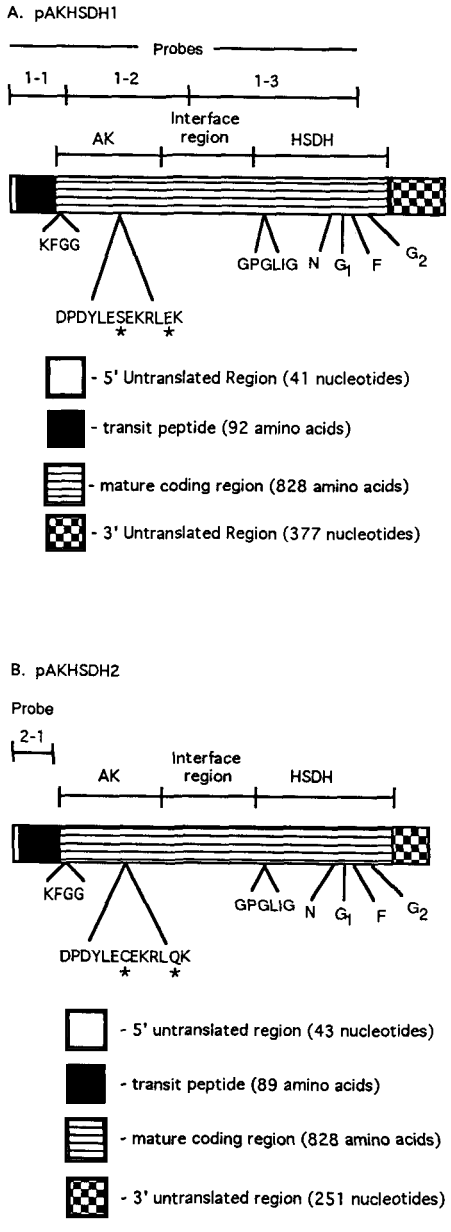

Figure 2. Molecular genetic structures of maize AK-HSDH. A, Structure of pAKHSDH1 and some deduced amino acids and the deduced domains of AKI-HSDHI (the AK domain spans amino acids 93–341, the interface region spans amino acids 342–566, and the HSDH domain spans amino acids 567–920). The 13-amino acid peptide sequence (DPDYLESEKRLEK) deduced from pAKHSDH1 was used to raise antibodies. The Lys-Phe-Gly-Gly (KFGG) sequence is positioned at amino acids 98 to 101. The putative NADPH-binding domain (Gly-X-Gly-X-X-Gly motif) is positioned at amino acids 568 to 573. The locations of the sequences that correspond to the consensus N (amino acids 670–681), G_1 (amino acids 704–712), F (amino acids 741–745), and G_2 (amino acids 787–792) motifs of prokaryotic two-component regulators are indicated. Amino acids are numbered from the first Met. 1–1, 1–2, and 1–3 are the pAKHSDH1 probes used for DNA and RNA gel blot hybridizations. B, Structure of pAKHSDH2 and some deduced amino acids and the deduced domains of AKII-HSDHII (the AK domain spans amino acids 90–338, the interface region spans amino acids 339–563, and the HSDH domain spans amino acids 564–917). The 13-amino acid peptide sequence (DPDYLECEKRLQK) has two amino acid residue differences compared to the same region deduced from pAKHSDH1. The Lys-Phe-Gly-Gly (KFGG) sequence is positioned at amino acids 95 to 98. The putative NADPH-binding domain (Gly-X-Gly-X-X-Gly motif) is located at amino acids 565 to 570. The locations of the sequences that correspond to the consensus N (amino acids 667–678), G_1 (amino acids 701–709), F (amino acids 738–742), and G_2 (amino acids 784–789) motifs of two-component regulators are indicated. Amino acids are numbered from the first Met. 2–1 is the pAKHSDH2 probe used for DNA gel blot hybridizations.

respectively) compared to typical transit peptides, which contain approximately 28% (von Heijne et al., 1989).

AKI-HSDHI and AKII-HSDHII exhibit some interesting features. All AK enzymes examined from both eukaryotes and prokaryotes have a four-amino acid, Lys-Phe-Gly-Gly, sequence near the amino terminus of the mature protein (Fig. 2). Chen et al. (1993) suggested that this region may function as the catalytic site for AK activity. The known AK-HSDH bifunctional and monofunctional AK enzymes from prokaryotic species also contain an inactive interface region (Belfaiza et al., 1984) (Fig. 2). Based on comparisons to the *E. coli* AKI-HSDHI enzyme, the interface region spans approximately amino acids 342 to 566 and 339 to 563 of maize AKI-HSDHI and AKII-HSDHII, respectively. Furthermore, the HSDH domains of AKI-HSDHI and AKII-HSDHII exhibit the highest amino acid identity with the corresponding domain in the enzymes compared (Table I), indicating, as in carrot AK-HSDH (Weisemann and Matthews, 1993), that the HSDH domain is more highly conserved than the AK domain. Finally, the NADPH-binding domain for the HSDH domain has been proposed to consist of a Gly-X-Gly-X-X-Gly motif (Parsot and Cohen, 1988). Both AKI-HSDHI and AKII-HSDHII contain this putative binding domain (Fig. 2).

In the HSDH domain of AKI-HSDHI and AKII-HSDHII (Fig. 2) we have identified four sequence motifs that are generally conserved in transmitter modules of some prokaryotic two-component regulator proteins (Parkinson and Kofoid, 1992) (Fig. 4). The bacterial transmitter modules comprise sequence motifs referred to as H, N, G_1, F, and G_2 based on their characteristic amino acid. In AKI-HSDHI and AKII-HSDHII, sequence similarity was observed for the consensus N, G_1, F, and G_2 motifs and a proposed transmitter protein DctB from *Rhizobium leguminosarum* (Ronson et al., 1987; Parkinson and Kofoid, 1992); however, the H motif appears to be either missing or diverged significantly from the prokaryotic consensus sequences. The order of the four domains in AKI-HSDHI and AKII-HSDHII was also consistent with prokaryotic transmitter modules. Two-component regulators regulate the adaptive responses to environmental stimuli primarily through phosphorylation mechanisms (Parkinson and Kofoid, 1992). For example, the *R. leguminosarum* DctB protein is thought to act as a receptor for C4-dicarboxylates and to regulate C4-dicarboxylate transport genes (Ronson et al., 1987). The G_1, F, and G_2 motifs are thought to participate in nucleotide binding. Sequence conservation of AKI-HSDHI and AKII-HSDHII with prokaryotic consensus sequences and

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                     +      ++       +++   +
Z. mays AKI-HSDHI   MRSLTVASRHPGAAFSTRRRPLLHPA
                    |  | |    | ||   | ||
Z. mays AKII-HSDHII MQGLAVSCQLPPAAAAARWRP--RAS
                                     + +   +

    +-    +  + -+  -       +  +  +  + -  +
 AAGRDSTFQRCWRWEKTQDSSFGSSLRTSRLPRTVHGDILKNLLA
    |    | ||  |  ||   |  ||        |     | |
 SSNREAVLQ-CWKYELSQDHYLGGPLRIGQSQGSLHRHRSTNFLR
    +-       + -   -+      +        ++++     +

         -  -   -  + -
 PTAGAVSVEQAEAIADLPKGD / MWSVHKFGGT
 | | | |||| |    |||||   ||||||||||
 PAAAAISVEQDEVNTYLPKGD / MWSVHKFGGT
         - --      + -
                         |  | |||||
E. coli AKI-HSDHI........M-RVLKFGGT
                         | ||||||||
S. marcescens AKI-HSDHI..M-RVLKFGGT
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Figure 3. Transit peptide sequences and putative mature enzyme start sites of AKI-HSDHI and AKII-HSDHII. Comparison of the transit peptide sequences from AKI-HSDHI and AKII-HSDHII. Positively and negatively charged amino acids are noted. The "/" indicates the possible cleavage site and the putative start of the mature AK-HSDH subunits based on the comparison to the first nine amino acids of *E. coli* and *S. marcescens* AKI-HSDHI (Katinka et al., 1980; Omori et al., 1993).

corresponding motifs from DctB (Fig. 4) suggests novel possibilities for regulation of AK-HSDH.

AK-HSDH cDNAs Encode a Thr-Sensitive Bifunctional Enzyme

To verify the identity of the cDNA clones, biochemical and immunological experiments were conducted. Antibodies were raised against a synthetic 14-amino acid peptide (CDPDYLESEKRLEK) deduced from the amino acid sequence of the AK domain of pAKHSDH1 (Fig. 2) conjugated to keyhole limpet hemocyanin. These antibodies recognized the synthetic AK-derived peptide in protein gel blots of BSA-conjugated peptide (data not shown).

AK and HSDH activities from Black Mexican Sweet maize suspension cells were bound to a Blue Sepharose column and eluted stepwise with 0.5 mM NADPH (Table II). Only Thr-sensitive AK (and no Lys-sensitive AK activity) was recovered in the fraction bound by Blue Sepharose; however, the HSDH activity was not completely Thr sensitive (Table II). Thr-sensitive AK (100% Thr sensitive) was purified approximately 61-fold. HSDH activity (72% Thr sensitive) was purified approximately 50-fold (Table II). AK and HSDH activities were visualized at the same position on nondenaturing PAGE gels (Fig. 5). Both activities exhibited Thr inhibition (Fig. 5; Table II). The peptide-specific antibodies cross-reacted with a protein that migrated to the same position as the Thr-sensitive AK and Thr-sensitive HSDH activities on a nondenaturing PAGE gel blot (Fig. 5). These data are consistent with the hypothesis that the pAKHSDH1 cDNA encodes an AK-HSDH bifunctional enzyme.

The AKI-HSDHI-derived antibodies also cross-reacted with a 89,000-D protein on an SDS-PAGE western blot of partially purified AK-HSDH (Fig. 6), which is similar to the reported molecular mass for a subunit of maize Thr-sensitive HSDH (Walter et al., 1979; Krishnaswamy and Bryan, 1986) and to the predicted mature polypeptide encoded by pAKHSDH1 and pAKHSDH2. AKII-HSDHII differs at two residues from the AKI-HSDHI peptide that was used to raise antibodies (Fig. 2). It was not determined whether the subunit encoded by pAKHSDH2 cross-reacts with the antibody derived from the deduced amino acid sequence of pAKHSDH1. However, the high amino acid identity between AKI-HSDHI and AKII-HSDHII indicates that pAKHSDH2 also encodes an AK-HSDH subunit.

Our biochemical and immunological results (Table II; Fig. 5) suggest that both the AK and HSDH activities of the maize bifunctional AK-HSDH are feedback inhibited by Thr. Walter et al. (1979) demonstrated that Blue Sepharose affinity chromatography separates the Thr-insensitive HSDH (unbound) from Thr-sensitive HSDH (bound). The HSDH activity in our bound fraction was 72% inhibited by 5 mM Thr (Table II). One explanation for the presence of Thr-insensitive HSDH in this preparation could be that Thr-sensitive HSDH has undergone a ligand-induced transition to a Thr-insensitive state as previously shown in maize (Krishnaswamy and Bryan, 1983a, 1983b).

Transcript Size and Expression of the AK-HSDH Genes

RNA gel blots hybridized with probe 1–2 (Fig. 2) of pAKHSDH1 identified a 3.2-kb transcript in Black Mexican Sweet suspension culture cells, immature embryos, and en-

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                 N            G1      F      G2
              motif          motif motif  motif

                              T  T        T
            V  VVV VV    V  D S VS V Q    P
            M  MMM MM    M  Q P MG M E    G
            L  LLL LL    L  E G IA L D    A
Consensus
sequence    I-QIIINII-NA I-DNGAGLP IFNPF GSGLGL
            |  |  |   |      ||||| ||| |  ||| |
AKII-HSDHII IHVITPNKKANS EATVGAGLP IFNNF ESGLRL
            |||||||||||| ||||||||| ||||| |||| |
AKI-HSDHI   IHVITPNKKANS EATVGAGLP IFNNF ESGLGL
                  |   |   |  ||| |  |  | ||||||
DctB        LEQVLINLLQNA VADNGPGIP LFTPF ESGLGL

AK-HSDH
identity
with DctB        17          44       40  100, 83^a

AK-HSDH
homology
with DctB        42          67       60  100, 83^a
```

Figure 4. Comparisons of maize AK-HSDH sequences to the consensus sequence from the N, G_1, F, and G_2 motifs derived from prokaryotic two-component regulators (Parkinson and Kofoid, 1992) and the *R. leguminosarum* regulatory protein DctB (Ronson et al., 1987). Amino acids listed above the consensus sequence are possible in the motifs, and dashes indicates wild card positions where less than 50% family conservation was observed (Parkinson and Kofoid, 1992). A superscript a ([a]) indicates AKII-HSDHII percentage identity with DctB.

Table II. *Feedback inhibition properties of purified maize AK-HSDH*

Purification of AK and HSDH from Black Mexican Sweet suspension cells (75 g). Specific activity for AK was 0.0196 and 1.2 nkatal mg^{-1} from the $(NH_4)_2SO_4$ and Blue Sepharose preparations, respectively. Fold purification of AK was calculated for Thr-sensitive AK. Specific activity for HSDH was 1,554 and 78,000 from the $(NH_4)_2SO_4$ and Blue Sepharose preparations, respectively. One unit of HSDH activity was defined as the change in A_{340} of 0.001 min^{-1}. Fold purification of HSDH was calculated for total HSDH. Inhibition properties of AK and HSDH were determined by performing the assays in 5 mM Thr and 5 mM Lys.

Steps	AK			HSDH	
	Percentage Thr inhibition	Percentage Lys inhibition	Fold purification	Percentage Thr inhibition	Fold purification
$(NH_4)_2SO_4$	21	50		53	
Blue Sepharose	100	0	61	72	50

dosperm tissue (Fig. 7) and in 7-d-old leaf tissue (data not shown). RNA gel blots probed with fragment 1–3 also identified only a 3.2-kb transcript in these tissues (data not shown). These data demonstrate that the AK-HSDH bifunctional enzyme is encoded by transcripts of a single size that correspond to the size of the cDNAs.

Expression of the AK-HSDH mRNA species appeared highest in embryos with slightly lower levels in suspension cells and low levels in endosperm tissue (Fig. 7). Blots probed with gene-specific probes from pAKHSDH1 and pAKHSDH2 (probes 1–1 and 2–4, respectively) did not reveal tissue-specific expression among the four tissues tested (data not shown). However, blots probed with pAKHSDH1 appeared to have a substantially higher transcript level than blots probed with pAKHSDH2. This result is currently being investigated further to determine whether the genes are expressed differentially.

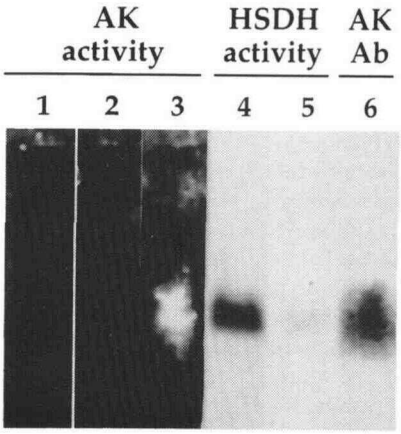

Figure 5. Co-migration of AK and HSDH activities on nondenaturing gels and verification of maize AK-HSDH cDNAs. Partially purified AK (1.8 pkatal [pmol/s] of activity in each lane) was electrophoresed in lanes 1 to 3. AK assays with (lane 3) and without Asp (lane 2) indicated the location of AK activity (white precipitate, lane 3). AK assays with 5 mM Thr (lane 1) demonstrated that this enzyme preparation contains Thr-sensitive AK. HSDH (8 units of activity in each lane) was electrophoresed in lanes 4 to 6. HSDH assays with (lane 5) and without 5 mM Thr (lane 4) demonstrated that this enzyme preparation contains a Thr-sensitive HSDH that migrates at the same position as Thr-sensitive AK. Lane 6 was blotted to an Immobilon-P membrane and reacted with the maize AKI-HSDHI peptide-derived antibody. The AK antibody cross-reacted with a protein at the same position as the Thr-sensitive AK and HSDH activities, indicating that pAKHSDH1 encodes a subunit of AK-HSDH.

Chromosomal Locations of AK-HSDH

The chromosomal locations of the genes that encode subunits of AK-HSDH were determined using a maize immortalized F_2 population (Gardiner et al., 1993). DNA gel blots were hybridized with gene-specific probes 1–1 and 2–1 (Fig. 2) from pAKHSDH1 and pAKHSDH2, respectively. Polymorphic bands were scored and entered into the University of Missouri RFLP data base and run on the MAPMAKER computer program (Lander et al., 1987) to determine the map location with respect to the core RFLP map (Gardiner et al., 1993). pAKHSDH1 was positioned to chromosome 4S between UMC191Gpc1 and UMC201NR at 7.5 and 2.7 cM,

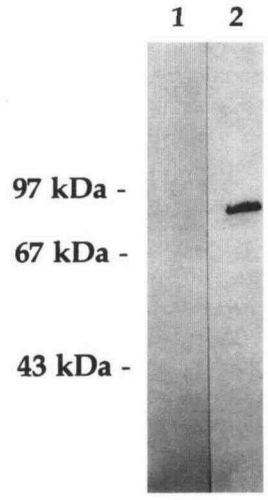

Figure 6. Molecular mass determination of AK-HSDH. Partially purified AK-HSDH (Blue Sepharose enzyme preparation) was electrophoresed on SDS-PAGE (lanes 1 and 2) and blotted. The AKI-HSDHI-derived antibody cross-reacted with a 89-kD subunit protein (lane 2). Control (preimmune) serum (lane 1) did not cross-react with any proteins.

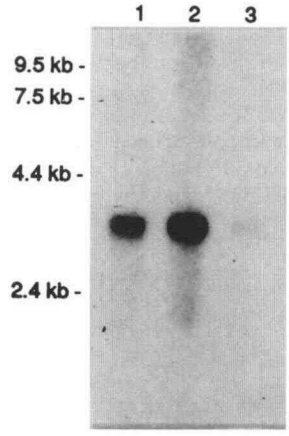

Figure 7. RNA gel blot analysis of AK-HSDH. Poly(A)$^+$ mRNA (2 mg) from Black Mexican Sweet suspension cells (lane 1; 7 d after subculture), B73 embryo (lane 2; 20 d after pollination), and endosperm (lane 3; 20 d after pollination) were transferred to a Nytran membrane and probed with fragment 1–2 from pAKHSDH1. This probe hybridized to a 3.2-kb transcript in these tissues.

respectively. pAKHSDH2 was positioned to chromosome 2L between UMC055 and UMC139 at 3.6 and 4.9 cM, respectively. Nonspecific probes for pAKHSDH3 detected polymorphisms only on chromosomes 4S and 2L in the same locations as pAKHSDH1 and pAKHSDH2. These blots also contained other monomorphic bands; therefore, an alternate location for pAKHSDH3 may be possible. A gene-specific probe for pAKHSDH3 did not detect a polymorphism between the F_2 parents, Tx303 and Co159, with 20 restriction enzymes, and it has not been possible to determine the map location of pAKHSDH3.

DISCUSSION

The results demonstrate the presence of at least three expressed genes encoding subunits of the Thr-sensitive AK-HSDH bifunctional enzyme in maize. Thr-sensitive HSDH makes up the major portion of HSDH activity in maize (Walter et al., 1979), suggesting an important role for this enzyme in amino acid biosynthesis. Recent biochemical separation of maize AK-HSDH from Lys-sensitive, monofunctional AK (Azevedo et al., 1992a, 1992b) and characterization of AK-HSDH clones from carrot and *Arabidopsis* (Weisemann and Matthews, 1993; Ghislain et al., 1994) demonstrated the presence of the bifunctional enzyme in plants, but little was known about the genes encoding AK-HSDH in maize. Our analyses indicated that two *ak-hsdh* genes were genetically distinct from the *ask1* and *ask2* genes that encode Lys-sensitive AK. Dominant mutations in the *ask* genes cause AK to become Lys insensitive, which leads to Thr overproduction in maize kernels (Dotson et al., 1990a). *ask1* is located on chromosome 7S (Azevedo et al., 1990), genetically distinct from *ak-hsdh* map locations on chromosome 2L and 4S. *ask2* was positioned on chromosome 2L, linked to but genetically distinct from the map location for pAKHSDH2 (Muehlbauer et al., 1994). The genetic data also showed that at least five genes encode AK, three for AK-HSDH and two for monofunctional AK. Furthermore, RNA gel blots hybridized under low-stringency conditions using AK (1–2 probe) and HSDH (1–3 probe) probes (Fig. 2) from pAKHSDH1 did not hybridize to transcripts smaller than 3.2 kb, indicating little or no homology to the expected transcripts for monofunctional AK or HSDH (data not shown). The lack of smaller hybridizing bands on the RNA blots suggests that AK-HSDH has diverged significantly from both the monofunctional AK and HSDH. In contrast, carrot RNA blots probed with the carrot AK-HSDH cDNA exhibited faint hybridization to transcripts of the size expected for a monofunctional AK or HSDH (Weisemann and Matthews, 1993).

The physiological role of the AK-HSDH bifunctional enzyme in plants is not well understood because it was just recently identified. Of specific interest is the putative role of the AK domain of the bifunctional enzyme. Previous physiological research concentrated on the Thr-sensitive HSDH domain without the knowledge that AK activity was present. Thr sensitivity of *E. coli* AKI-HSDHI is exerted by the AK domain of the enzyme (Truffa-Bachi et al., 1974). Point mutations identified in the intermediary domain near the AK domain of the *S. marcescens* AKI-HSDHI enzyme rendered both enzymes Thr insensitive (Omori et al., 1993). Deletion mutants of *S. marcescens* AKI-HSDHI in the HSDH domain altered the multimer formation of the enzyme and decreased the AK Thr sensitivity (Omori et al., 1993). Conformational transitions of the AK domain of *E. coli* AKI-HSDHI were detected by altered cross-reactivity with anti-AK domain antibodies when the bifunctional enzyme was incubated in the presence or absence of Thr. The experiments on *S. marcescens* and *E. coli* AK-HSDH indicate that transitions in AK conformation or alteration of multimer formation may modulate Thr sensitivity of the holoenzyme. It is not known whether similar mechanism(s) are involved in Thr inhibition of plant AK-HSDH. However, similar mechanisms may account for transitions in subunit configurations and modulation of Thr sensitivity reported in plants (Krishnaswamy and Bryan, 1983a, 1983b; Turano et al., 1990). Maize AK-HSDH was shown to be Thr sensitive (Table II; Fig. 5; Azevedo et al., 1992b), at least indicating that the AK domain may be active in Thr inhibition of the enzyme.

Thr-sensitive AK makes up only a minor portion (20% in this study) of the total cellular AK activity; the majority is due to Lys-sensitive AK. The presence of Lys-sensitive AK likely would compensate for the low level of Thr-sensitive AK if the enzyme were inhibited by Thr. Thr-sensitive HSDH appears to make up a major portion of total HSDH activity (53% in our assay conditions in this study), suggesting an essential role for this activity in plastid-localized Thr synthesis (Bryan et al., 1977). The role of Thr-insensitive HSDH in amino acid biosynthesis is not understood (Bryan, 1990b). In light of these observations, it is most likely that lethal Met starvation, induced by the inclusion of equimolar concentrations of Lys and Thr in plant tissue culture media (Gengenbach et al., 1992), is due to complete inhibition of Lys-sensitive monofunctional AK and Thr-sensitive AK activity of AK-HSDH. It is less clear why *Ask* mutants of AK with reduced Lys feedback inhibition overproduce free Thr (Dotson et al., 1990a). Perhaps homoserine is translocated into the cytoplasm, where a nonplastidic Thr-insensitive HSDH

might be located or perhaps Thr sensitivity of plastidic AK-HSDH is somehow relaxed.

Bryan (1990b) reported that the degree of Thr sensitivity of HSDH is regulated by environmental conditions. In in vitro assays, Thr-sensitive HSDH is inhibited by micromolar concentrations of Thr in low pH incubation medium representing conditions in unilluminated chloroplasts. In media representing illuminated chloroplasts (alkaline pH), Thr inhibition of HSDH is markedly reduced. Presumably, reduced Thr feedback inhibition of HSDH in illuminated chloroplasts permits amino acid biosynthesis to proceed at an increased rate, suggesting a direct link between light-dependent carbon and nitrogen assimilation and amino acid biosynthesis (Bryan, 1990b). It is possible that in the Thr-overproducing *Ask* mutants similar modulations of Thr sensitivity of AK-HSDH would permit Thr overproduction. The activity of Thr-sensitive AK has not been examined under the conditions used by Bryan (1990b) to investigate Thr-sensitivity of HSDH conferred by AK-HSDH. Therefore, its putative role in modulating HSDH activity of the bifunctional enzyme during light/dark transitions has not been determined.

Giovanelli et al. (1989a, 1989b) have shown that Lys and Thr feedback inhibition of AK may not be the major factor limiting Thr synthesis in *Lemna paucicostata*. However, the maize (Dotson et al., 1990a), barley (*Hordeum vulgare*) (Bright et al., 1982a, 1982b), and tobacco (*Nicotiana sylvestris*) (Frankard et al., 1991) *Ask* mutants (Lys-insensitive AK mutants) and the transgenic tobacco plants containing a Lys-insensitive AK from *E. coli* (Shaul and Galili, 1992; Karchi et al., 1993) all exhibited the Thr-overproducing phenotype. These comparisons of mutant and wild-type plants provide compelling evidence for in vivo regulation of Thr synthesis in wild-type versions of these species. Perhaps the regulation of Thr synthesis differs in *Lemna*.

One hypothesis for the role of AK in AK-HSDH is that the AK domain functions to perceive cellular Thr concentration and thereby regulate both AK and HSDH activity via a novel regulatory mechanism. Our proposed model is based on the presence of four conserved sequence motifs characteristic of prokaryotic two-component regulatory systems (Parkinson and Kofoid, 1992) (Fig. 4). Sequences with homology to the H motif from two-component regulatory systems were not observed in maize AKI-HSDHI and AKII-HSDHII. However, there are examples of prokaryotic two-component regulatory modules that do not have a conserved H motif (Parkinson and Kofoid, 1992). It should be noted that the monofunctional HSDH enzyme from yeast and bifunctional AK-HSDH enzymes from prokaryotes, carrot, and *Arabidopsis* also contain homology to the N, G_1, F, and G_2 motifs. Chang et al. (1993) reported that the *Arabidopsis* ETR1 protein (ethylene response) shows significant similarity to prokaryotic two-component regulators, demonstrating their presence in plants. In two-component regulatory systems, signal perception is mediated by an amino-terminal domain referred to as the sensor. Signal perception results in autophosphorylation of His in the H motif by a C-terminal His kinase activity conferred by the region containing the N, G_1, F, and G_2 motifs. The phosphate is transferred from the phosphorylated His of the sensor to a specific Asp residue on a response regulatory module, which may or may not be on the same protein, to either activate or inactivate function of the regulatory module. Allosteric regulation of maize AK-HSDH, or perhaps all AK-HSDH bifunctional enzymes, could involve a similar phosphorylation mechanism to regulate amino acid biosynthesis. In our model, the AK domain may perceive cellular Thr concentration. Under certain conditions, autophosphorylation of AK-HSDH (presumably at an Asp) may involve the N, G_1, F, and G_2 motifs in the HSDH domain. Autophosphorylation may modulate Thr sensitivity of HSDH under certain physiological conditions.

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LITERATURE CITED

Aarnes H, Rognes SE (1974) Threonine-sensitive aspartate kinase and homoserine dehydrogenase from *Pisum sativum*. Phytochemistry **13:** 2717–2724

Azevedo RA, Arana JL, Arruda P (1990) Biochemical genetics of the interaction of lysine plus threonine resistant mutant *Ltr*1* with *opaque-2* maize mutant. Plant Sci **70:** 81–90

Azevedo RA, Blackwell RD, Smith RJ, Lea PJ (1992a) Three aspartate kinase isoenzymes from maize. Phytochemistry **31:** 3725–3728

Azevedo RA, Smith RJ, Lea PJ (1992b) Aspartate kinase regulation in maize: evidence for co-purification of threonine-sensitive aspartate kinase and homoserine dehydrogenase. Phytochemistry **31:** 3731–3734

Belfaiza J, Fazel A, Muller K, Cohen GN (1984) *E. coli* aspartokinase II-homoserine dehydrogenase II polypeptide chain has a triglobular structure. Biochem Biophys Res Commun **123:** 16–20

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bright SWJ, Kueh JSH, Franklin J, Rognes SE, Miflin BJ (1982a) Two genes for threonine accumulation in barley seeds. Nature **299:** 278–279

Bright SWJ, Miflin BJ, Rognes SE (1982b) Threonine accumulation in the seeds of a barley mutant with an altered aspartate kinase. Biochem Genet **20:** 229–243

Bryan JK (1990a) Advances in the biochemistry of amino acid biosynthesis. *In* BJ Miflin, PJ Lea, eds, The Biochemistry of Plants, Vol 16. Academic Press, New York, pp 161–195

Bryan JK (1990b) Differential regulation of maize homoserine dehydrogenase under physiological conditions. Plant Physiol **92:** 785–791

Bryan JK, Lissik EA, Matthews BF (1977) Changes in enzyme regulation during growth of maize. III. Intracellular localization of homoserine dehydrogenase in chloroplasts. Plant Physiol **59:** 673–679

Cassan M, Parsot C, Cohen GN, Patte JC (1986) Nucleotide sequence of *lysC* gene encoding the lysine-sensitive aspartokinase III of *Escherichia coli* K12. J Biol Chem **261:** 1052–1057

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science **262:** 539–544

Chen N-Y, Hu F-M, Paulus H (1987) Nucleotide sequence of overlapping genes for the subunits of *Bacillus subtilis* aspartokinase II and their control regions. J Biol Chem **262:** 8787–8798

Chen N-Y, Jiang S-Q, Klein DA, Paulus H (1993) Organization and nucleotide sequence of the *Bacillus subtilis* diaminopimelate operon, a cluster of genes encoding the first three enzymes of diaminopimelate synthesis and dipicolinate synthase. J Biol Chem **268:** 9448–9465

Chisholm D (1989) A convenient moderate-scale procedure for obtaining DNA from bacteriophage lambda. Biotechniques **7:** 21–23

Dotson SD, Frisch DA, Somers DA, Gengenbach BG (1990a) Lysine-insensitive aspartate kinase in two threonine-overproducing mutants of maize. Planta **182:** 546–552

Dotson SD, Somers DA, Gengenbach BG (1989) Purification and characterization of lysine-sensitive aspartate kinase from maize cell cultures. Plant Physiol **91:** 1602–1608

Dotson SD, Somers DA, Gengenbach BG (1990b) Kinetic studies of lysine-sensitive aspartate kinase purified from maize. Plant Physiol **93:** 98–107

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem **132:** 6–13

Frankard V, Ghislain M, Negrutiu I, Jacobs M (1991) High threonine producer mutant of *Nicotiana sylvestris* (Spegg. and Comes). Theor Appl Genet **82:** 273–282

Gardiner JM, Coe EH, Melia-Hancock S, Hoisington DA, Chao S (1993) Development of a core RFLP map in maize using an immortalized F_2 population. Genetics **134:** 917–930

Gengenbach BG, Somers DA, Keith RA, Muehlbauer GJ, Sellner JM, Bittel DC, Shaver JM (1992) Cellular and molecular genetic regulation of the synthesis of lysine, methionine, and threonine-the aspartate family amino acids. *In* BK Singh, HE Flores, JC Shannon, eds, Biosynthesis and Molecular Regulation of Amino Acids in Plants. American Society of Plant Physiologists, Rockville, MD, pp 195–207

Gengenbach BG, Walter TJ, Green CE, Hibberd KA (1978) Feedback regulation of lysine, threonine, and methionine biosynthetic enzymes in corn. Crop Sci **18:** 472–476

Ghislain M, Frankard V, Vandenbossche D, Matthews BF, Jacobs M (1994) Molecular analysis of the aspartate kinase-homoserine dehydrogenase gene from *Arabidopsis thaliana*. Plant Mol Biol **24:** 835–851

Giovanelli J, Mudd SH, Datko AH (1989a) Aspartokinase of *Lemna paucicostata* Hegelm. 6746. Plant Physiol **90:** 1577–1583

Giovanelli J, Mudd SH, Datko AH (1989b) Regulatory structure of the biosynthetic pathway for the aspartate family of amino acids in *Lemna paucicostata* Hegelm. 6746, with special reference to the role of aspartokinase. Plant Physiol **90:** 1584–1599

Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Hitchcock MJM, Hodgson B (1976) Lysine- and lysine-plus-threonine- inhibitable aspartokinases in *Bacillus brevis*. Biochem Biophys Acta **445:** 350–363

Karchi H, Shaul O, Galili G (1993) Seed-specific expression of a bacterial desensitized aspartate kinase increases the production of seed threonine and methionine in transgenic tobacco. Plant J **3:** 721–727

Katinka M, Cossart P, Sibilli L, Saint-Girons I, Chalvignac MA, Le Bras G, Cohen GN, Yaniv M (1980) Nucleotide sequence of the *thrA* gene of *Escherichia coli*. Proc Natl Acad Sci USA **77:** 5730–5733

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Annu Rev Plant Physiol Plant Mol Biol **40:** 471–501

Krishnaswamy S, Bryan JK (1983a) Ligand-induced interconversions of maize homoserine dehydrogenase among different states. Arch Biochem Biophys **222:** 449–463

Krishnaswamy S, Bryan JK (1983b) Characterization of ligand-induced states of maize homoserine dehydrogenase. Arch Biochem Biophys **227:** 210–224

Krishnaswamy S, Bryan JK (1986) Use of monoclonal antibodies for the purification and characterization of the threonine-sensitive isozyme of maize homoserine dehydrogenase. Arch Biochem Biophys **246:** 250–262

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1:** 174–181

Muehlbauer GJ, Gengenbach BG, Somers DA, Donovan CM (1994) Genetic and amino acid analysis of two maize lysine-insensitive, threonine overproducing mutants. Theor Appl Genet (in press)

Omori K, Imai Y, Suzuki S, Komatsubara S (1993) Nucleotide sequence of the *Serratia marcescens* threonine operon and analysis of the threonine operon mutations which alter feedback regulation of both aspartokinase I and homoserine dehydrogenase I. J Bacteriol **175:** 785–794

Parkinson JS, Kofoid EC (1992) Communication modules in bacterial signaling proteins. Annu Rev Genet **26:** 71–112

Parsot C, Cohen GN (1988) Cloning and nucleotide sequence of the Bacillus subtilis *Homo* gene coding for homoserine dehydrogenase—Structural and evolutionary relationships with *Escherichia coli* aspartokinases-homoserine dehydrogenase I and dehydrogenase II. J Biol Chem **263:** 14654–14660

Rafalski JA, Falco SC (1988) Structure of the yeast HOM3 gene which encodes aspartokinase. J Biol Chem **263:** 2146–2151

Relton JM, Bonner PLR, Wallsgrove RM, Lea PJ (1988) Physical and kinetic properties of lysine-sensitive aspartate kinase purified from carrot suspension culture. Biochem Biophys Acta **953:** 48–60

Ronson CW, Astwood PM, Nixon BT, Ausubel FM (1987) Deduced products of C4-dicarboxylate transport regulatory genes of *Rhizobium leguminosarum* are homologous to nitrogen regulatory gene products. Nucleic Acids Res **15:** 7921–7934

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA **81:** 8014–8019

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shaul O, Galili G (1992) Threonine overproduction in transgenic tobacco plants expressing a mutant desensitized aspartate kinase of *Escherichia coli*. Plant Physiol **100:** 1157–1163

Thomas D, Barbey R, Surdin-Kerjan Y (1993) Evolutionary relationships between yeast and bacterial homoserine dehydrogenases. FEBS Lett **323:** 289–293

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Truffa-Bachi P, Veron M, Cohen GN (1974) Structure, function, and possible origin of a bifunctional allosteric enzyme, *Escherichia coli* aspartokinase I-homoserine dehydrogenase I. CRC Crit Rev Biochem **2:** 379–415

Turano FJ, Jordan RL, Matthews BF (1990) Immunological characterization of in vitro forms of homoserine dehydrogenase from carrot suspension cultures. Plant Physiol **92:** 395–400

Van Regermortel MHV, Briand JP, Muller S, Plau S (1988) Synthetic Polypeptides as Antigens: Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier, Amsterdam, The Netherlands

von Heijne G, Steppuhn J, Herrmann RG (1989) Domain structure of mitochondrial and chloroplast targeting peptides. Eur J Biochem **180:** 535–545

Walter TJ, Connelly JA, Gengenbach BG, Wold F (1979) Isolation and characterization of two homoserine dehydrogenases from maize suspension cultures. J Biol Chem **254:** 1349–1355

Weisemann JM, Matthews BF (1993) Identification and expression of a cDNA from *Daucus carota* encoding a bifunctional aspartokinase-homoserine dehydrogenase. Plant Mol Biol **22:** 301–312

Wilson BJ, Gray AC, Matthews BF (1991) Bifunctional protein in carrot contains both aspartate kinase and homoserine dehydrogenase activities. Plant Physiol **97:** 1323–1328

Zakin MM, Duchange N, Ferrara P, Cohen GN (1983) Nucleotide sequence of the *metL* gene of *Escherichia coli*. J Biol Chem **258:** 3029–3031